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EVALUATION OF BACTERIAL INVOLVEMENT IN AN EPISODE OF NEONATAL CALF DIARRHEA

Aurel VASIU, Emöke PÁLL, Marina SPÎNU, Sergiu Dan ZĂBLĂU, Emilia UNGUREANU, Monica Ioana SUĂTEAN, Gheorghe Florinel BRUDAȘCĂ, Diana Ioana OLAH

University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Faculty of Veterinary Medicine, 3-5 Mănăștur Street, 400372, Cluj-Napoca, Romania

Corresponding author: emoke.pall@usamvcluj.ro

Abstract

Diarrhea and digestive disorders represent one of the leading causes of mortality in calves during the first days of life. This study aimed to establish the level of antibiotic resistance of the flora identified in calves with digestive problems and also in their environment. Bălțată Românească calves aged up to three weeks, showing an increased morbidity/mortality from a farm in Brașov county, as well as their habitat were sampled. Eight antimicrobials used in ruminants and on this specific farm (penicillin, streptomycin, amoxicillin, gentamicin, oxytetracycline, tulathromycin, cefaclor, trimethoprim/ sulfamethoxazole) were tested for their efficacy against the bacterial isolates. The samples were subjected to standard microbiological examinations; subsequently, strains such as Escherichia fergusoni, Escherichia hermannii, Shigella dysenteriae, Proteus penneri, Morganella morganii ssp. siboni, Escherichia coli, Escherichia vulneris, with potentially high pathogenicity were identified. The highest antibacterial efficacy was observed with gentamicin. The MAR (multiple antibiotic resistance) index exceeded the value of 0.2 for each of the tested strains, indicating a high level of antibiotic resistance of the isolated bacterial population, thus supporting the inducive role of isolated species in the clinical episodes.

Key words*: antibiotic resistance, neonatal diarrhea, calves, E. coli.*

INTRODUCTION

Neonatal calf diarrhea (NCD) is a significant cause of calf mortality, reduced growth and increased age, difficulty in first calving, leading to an economic loss worldwide (Windeyer et al., 2014).

It is a multi factorial disease entity that can have serious financial and welfare implications and is one of the most prevalent diseases seen in calves up to 3 months (Fentie et al., 2020; Yimer et al., 2015).

The disease may be triggered by both infectious and non-infectious factors - the individual characteristics of the calf, veterinary treatments applied, management of the herd, and environmental factors (Bendali et al., 1999).

Infectious agents are undoubtably the main cause of mortality. Among the numerous infectious agents causing NCD, rotavirus, coronavirus, *Escherichia coli* enterotoxin K99/F5, and *Cryptosporidium parvum* spp. are recognized as the four most encountered pathogens (Naylor, 2009). The aim of this study was to evaluate the degree of antibiotic resistance of Gram-negative microorganisms isolated from different areas in the farm and from diseased or healthy calves, together with the effectiveness of disinfection in controlling the bacterial population on farm. At the time of sampling, an elevated rate of mortality was seen in calves aged between 3 and 15 days, showing signs of severe diarrhea.

Farm and animals

The farm is located in Brașov county and at the time of the study comprised a herd of 70 adult cattle and 20 heifers of Bălțată Românească breed. Fatteners are also raised until the age of 11-12 months, when they are culled at an average weight of 400 kg. In addition, the owner had bought and introduced calves from a few other farms in the proximity. There were about 100 calves on the farm, 70 bulls and 30 females. The farm has two stables: a building with the capacity of accommodating 50 cows, dating from the communist era, and new stable built in 2010 which houses heifers and calves; this stable also has a well bounded extension in

which the owner keeps some of his sheep. The farmer also owns a flock of 300 sheep, Țurcană breed, pigs for family consumption and one horse.

The farm is situated on 250 ha of agricultural land, of which 150 ha of arable land and 100 ha of pasture. The source of fodder, corn for silage, oats, sunflower, soybeans, rye, alfalfa and other herbs crops are self-provided. The shelter is designed as a stable with medium stands, which are provided with tying chains at the edge of the manger. The floor is made of concrete, with sawdust or straw bedding. The lighting of the shelter is both natural and artificial. In the cold season, the animals have permanent housing, in the warm season they are taken out to pasture, and the pregnant heifers and the young are kept outside.

The calves are kept in boxes of 2-4 individuals in the first 15 days, then they are distributed into groups of 4-8, until weaning. After weaning, they are placed in boxes of 15-20 calves. Feeding is done by teat.

The mandatory sanitary actions had been previously carried through. All the animals had been subjected to the intradermal skin test for tuberculosis, anthrax vaccination, and to serological surveillance for enzootic bovine leukosis (EBL), bovine brucellosis and paratuberculosis.

Feed ratio

Animal feed consists of silage, alfalfa hay, hay and concentrated fodder composed of corn, wheat, oats, sunflower and soybeans (heat treated or soybean meal). Dairy cows are given a feed consisting of 50% silo, 25% alfalfa hay/grass, 20% flour and 5% premix mixed with flour (Table 1).

Table 1. Feed ratio according to age

	Days 0-7	Days 7-70	Days 71-130	Days 131-205	Days 206-330
Colostrum	6 L/day	-	-	-	-
Milk substituents (milk powder)	-	6 L/day	-	-	-
Concentrated fodder	-	A.l. (approx. intake 40 kg)	2 kg/day	8 kg/day	15 kg/day
Alfalfa hay	-	A.l.	A.l.	A.l.	A.l.
Mineral cubes	-	A.l.	A.l.	A.l.	A.l.
Corn silage	-	-	-	10 kg	15 kg
Water	-	A.l.	A.l.	A.l.	A.l.

A.l.= *ad libitum*

Description of morbid episodes

Once the number of cows on the farm increased, the farmer noticed significant rise in calves loss every year, from 5% to 30% in 4 years. In 2020, out of 80 calves of own production, about 50% showed digestive and respiratory problems. 50% of these died between 2 days and 6 weeks of age. The digestive symptoms included diarrhea, progressive weight loss, severe dehydration with enophthalmia, and permanent recumbency. Others conveyed respiratory signs, such as coughing and dyspnea. New calves were brought in and the number of calves reached 100. They were kept in boxes accommodating of 4-5 individuals each, for a 14 days quarantine. Antibiotics (tulathromycin and oxytetracycline) and vitamins were prophylactically administered when introduced into the herd.

In 2021, out of a total of 60 calves, about 40% showed signs of disease and approximately 40% died. 50 calves were subsequently introduced in order to restore the population. The same conduct was applied the next year, the calves were kept in boxes of 4-5 calves each, and upon introduction into the herd, they were prophylactically administered antibiotics (tulathromycin and amoxicillin) and vitamins. After weaning, they were placed in boxes of 15-20 individuals.

MATERIALS AND METHODS

Collection and transport of samples

Samples were taken either from healthy calves, from carcasses of from calves recovered from disease, from their stalls and from other farm areas difficult to reach (Table 2). These were collected using sterile swabs, moistened with 0.9% saline, which were subsequently introduced into Amies transport medium. The sampling from calves was done by inserting the tampon into the rectum and gently rotating the swab for 5 seconds. The samples were transported safely, at constant temperatures, protected from the action of sunlight and low temperatures. 8 samples were taken from animals/places at 28 days interval. The samples were transported to the Laboratory of Infectious Diseases within the Faculty of Veterinary Medicine, Cluj-Napoca, where they underwent bacteriological examination.

First sampling was done in March, as most parturitions occur during this month. The collection of the second round of samples was done within 4 weeks of the first sampling, under the conditions of application of chlorine and formalin-based disinfectants. It followed the same protocol, but included other calves with diarrhea or clinically healthy, aged between 2 and 4 weeks.

Table 2. Sampling sites and materials

Sample no.	First sampling source	Second sampling source	Sampled material
1.	Stall no. 1	Stall no. 1	Walls and hardly accesible areas
2.	2 days old calf with diarrhea	8 days calf with diarrhea	Rectum and feces
3.	10 days old healthy calf	12 days calf with diarrhea	Rectum and feces
4.	Stall no. 2	Stall no. 2	Walls and hardly accesible areas
5.	Stall no. 3	Stall no. 3	Walls and hardly accesible areas
6.	Stall no. 4	Stall no. 4	Walls and hardly accesible areas
7.	Stall no. 5	Stall no. 5	Walls and hardly accesible areas
8.	15 days old healthy calf	20 days old healthy calf	Rectum and feces

Microbiological examination

The samples were inoculated on meat broth and incubated for 24 hours at 37°C. Subsequently, they were transferred onto McConkey agar medium and subjected to the same incubation conditions.

The susceptibility to antibiotics was assessed by the Kirby Bauer disk-diffusion method. For the qualitative interpretation of the results, the diameters in mm of the inhibition zones were compared with the standard diameters established according to the antibiotic and its concentration by CLSI (Clinical and Laboratory Standards Institute), with the classification of bacterial strains into three distinct categories; sensitive, intermediate and resistant. We tested with 8 antibiotics recommended in this species and frequently used on the farm: streptomycin (Oxoid, 10 mcg/disk), amoxicillin (Oxoid, 30 mcg/disk), gentamicin (Oxoid, 30 mcg/disk), oxytetracycline (Oxoid, 30 mcg/disk), tulathromycin (Oxoid, 30 mcg/disk), cefaclor (Oxoid, 30 mcg/disk), trimethoprim/ sulfamethoxazole (Oxoid, 30 mcg/disk), penicillin (10 UI). For each bacterial strain, the Multiple Antibiotic Resistance (MAR) index was calculated according to Krumperman's formula. A MAR coefficient > 0.2 indicated exposure to high-risk sources of human or animal contamination, an environment with frequent use of antibiotics. In contrast, a MAR value <0.2 is considered to indicate a rare use or no use of antibiotics. The formula provides the ratio between the number of antibiotics to which the microorganism was resistant and the number of antibiotics to which it was exposed (Sandhu et al., 2016).

Biochemical identification

ENT 16 is a standardized identification system for common *Enterobacteriaceae* species. 16-18 species of microorganisms are included in the data base. ENT 16 kit consists of 16 microtiter strips in the classic 96-well format containing dehydrated substrates. The reconstruction of the substrates is done by inoculating the bacterial suspension. Color change occurs during incubation, due to the metabolic activity of microorganisms, which causes discoloration in individual wells. The test results are read visually based on a color scale. The identification results are read from the evaluation table or using the evaluation software.

RESULTS AND DISCUSSIONS

Following the described protocol, we identified of a number of 7 bacterial genera at the first, respectively 5 bacterial genera at the second sampling, performed after the disinfection. There was no statistically significant difference in the abundance of microorganisms between the two samplings (14 vs. 15 bacterial strains). The two *Escherichia* species together, *Hafnia alvei* and *Citrobacter braakii* accounted for 21.4%, followed by *Leclercia adecarboxylata* with 14.29% and *Morganella morganii* ssp.

sibonii, *Shigella dysenteriae* and *Proteus penneri* with 7.14% prevalence in the population isolated in the first sampling. *C. braakii* and *E. coli* accounted for 26.66% in the second sampling, followed by *Serratia odorifera* with 20%, *H. alvei* with 13.33%, and *Shigella dysenteriae* and *E. vulneris* with 6.66% (Table 3).

Table 3. Biochemical identification results of both samplings

Sampling site	First sampling	Second sampling
Walls and hardly accesible areas	1.1 *Morganella morganii ssp. sibonii*	1.1 *Citrobacter braakii* 1.2 *Hafnia alvei*
Rectum and feces	2.1 *Leclercia adecarboxylata* 2.2*Escherichia fergusonii*	2.1 *Citrobacter braakii* 2.2 *Serratia odorifera bv.2*
Rectum and feces	3.1 *Escherichia hermannii*	*3.1 Escherichia coli*
Walls and hardly accesible areas	4.1 *Leclercia adecarboxylata* 4.2 *Hafnia alvei* 4.3 *Citrobacter braakii*	4.1 *Shigella dysenteriae (gr. A)* 4.2 *Escherichia coli*
Walls and hardly accesible areas	5.1 *Shigella dysenteriae (gr. A)* 5.2 *Hafnia alvei* 5.3 *Citrobacter braakii*	5.1 *Serratia odorifera bv.1* 5.2 *Escherichia vulneris* 5.3 *Serratia odorifera bv.2*
Walls and hardly accesible areas	6.1 *Proteus penneri*	6.1 *Hafnia alvei bv.1*
Walls and hardly accesible areas	7.1 *Escherichia fergusonii*	7.1 *Escherichia coli* 7.2 *Citrobacter braakii*
Rectum and feces	8.1 *Citrobacter braakii* 8.2 *Hafnia alvei bv.1*	8.1 *Citrobacter braakii* 8.2 *Escherichia coli*

An increased bacterial load could be seen in calves and the environment. The most important bacteria blamed for the occurrence of neonatal diarrhea in calves in the first period of life belonged to the genus *Escherichia*. *E. coli*, *E. vulnerans* and *E. hermannii* were identified in the feces of calves, in the environment, in cracks in the walls and in inaccessible areas. They were also identified on the walls, areas continuously exposed to feces carrying various microorganisms. Bacteria were often found in areas more difficult to be reached by disinfectants, or where disinfectants were insufficiently applied. All the samples examined showed mixed microbial flora, in the majority of cases represented by the combination of Gram-positive and Gram-negative bacteria. We could not observe a decrease in the population of bacteria or a significant change in its variety between the first and second sampling, i.e. before and after the disinfection was applied.

The results showed that gentamicin was the most effective off all antibiotics tested on the bacterial populations identified at first sampling. It displayed antimicrobial activity against all agents identified on the farm, including *E. coli*, considered the most pathogenic. *E. coli* was resistant to penicillin, streptomycin, amoxicillin, tulathromycin, cefaclor, trimethoprim/sulfamethoxazole, amoxicillin/clavulanic acid.

Gentamicin had a very good action on bacterial populations isolated in the sampling which followed disinfection. Cefaclor, on the other hand, had the lowest efficiency. Tulatromycin, trimethroprim/sulfamethoxazole and amoxicillin also showed efficiency against on *E. coli*.

All of the isolates were attributed an elevated resistance to antibiotics, with a MAR index over 0.2 (Table 4). Extremely resistant were most of the microorganisms in the genera *Escherichia* and *Citrobacter*. As they were best represented in the samples, the finding was statistically defended.

Table 4. MAR index for each bacterial strain identified

Microorganism	MAR
First sampling	
1.1 *Morganella morganii* ssp. *sibonii*	0.375
2.1 *Leclercia adecarboxylata*	0.375
2.2 *Escherichia fergusonii*	0.75
3.1 *Escherichia hermannii*	0.75
4.1 *Leclercia adecarboxylata*	0.375
4. 2 *Hafnia alvei*	0.625
4.3 *Citrobacter braakii*	0.375
5.1 *Shigella dysenteriae* (gr. A)	0.625
5.2 *Hafnia alvei*	0.75
5.3 *Citrobacter braakii*	0.25
6.1 *Proteus penneri*	0.75
7.1 *Escherichia fergusonii*	0.75
8.1 *Citrobacter braakii*	0.50
8.2 *Hafnia alvei* bv. 1	0.625
Second sampling	
1.1 Citrobacter braakii	0.625
1.2 Hafnia alvei	0.75
2.1 Citrobacter braakii	0.75
2.2 Serratia odorifera bv. 2	0.75
3.1 Escherichia coli	0,5
4.1 Shigella dysenteriae (gr. A)	0.375
4.2 Escherichia coli	0.375
5.1 Serratia odorifera bv. 1	0.5
5.2 Escherichia vulneris	0.5
5.3 Serratia odorifera bv. 2	0.75
5.4 Escherichia vulneris	0.75
6.1 Hafnia alvei bv. 1	0.875
7.1 Escherichia coli	0.75
7.2 Citrobacter braakii	0,75
8.1 Citrobacter braakii	0.25
8.2 Escherichia coli	0.75

One of the underlying reasons for the occurrence of neonatal diarrhea is the failure to transfer passive immunity (Windeyer et al., 2014). Meanwhile it is possible that there is a management deficiency on farm, rendered in the poor hygiene - even bottle feeding of colostrum may be a source of *E. coli* contamination (Mohammed et al., 2018) - insufficient care for calves to suckle colostrum in the first few hours of life, vaccination was not considered for the prevention of the disease. In Romania, there are a few vaccines available, with reportedly good results in controlling this extremely prejudicial syndrome. Other results on the examination of samples collected from apparently healthy and diarrheic calves showed an incidence of 46.4%. The strains were sensitive to norfloxacin (80.7%) and resistant to ampicillin and cefotaxime (100%) (Mohammed et al., 2018). Another study on *E. coli* isolates showed a high frequency of EHEC and presence of a new phenotype, in both apparently healthy and diarrheic calves (Aref et al., 2018).

CONCLUSIONS

There was a significant load of microorganisms known to play a role in the etiology of neonatal calf diarrhea, most of which were extremely resistant to antimicrobials, which suggests the decisive role of bacteria in the etiology of this disease. The highest frequency of isolation belonged to *Escherichia* spp., which may be due to a deficiency of zoo hygiene in the farm. The results of the study indicated no decrease in the population of bacteria or any significant modification in its variety or antibiotic sensitivity between the first and second sampling, i.e. before and after the disinfection was applied. The extremely high level of antibiotic resistance found on the farm is alarming (Ungureanu et al., 2019). It reveals an intensive use of antibiotics and calls for the implementation of a strategy meant to limit the antimicrobial treatments. Molecular analysis is required in order to decide whether the bacterial strains of *E. coli* are highly pathogenic or play a lesser role in the course of the disease.

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